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TITLE: A Novel Screen for Suppressors of Breast Tumor Cell Growth Using an Oriented Random Peptide Library Method to Identify Inhibitors of the ErbB2 Tyrosine Kinase

PRINCIPAL INVESTIGATOR: Kermit L. Carraway, Ph.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, Massachusetts 02215

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Carraway, Kermit L., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Beth Israel Deaconess Medical Center
Boston, Massachusetts 02215

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We are developing a novel degenerate cyclic peptide library selection method to identify potential peptide antagonists of the ErbB2 receptor tyrosine kinase. Since the aberrant activation of the ErbB2 receptor is believed to be involved in the genesis or progression of a significant proportion of human breast tumors, a cyclic peptide antagonist that binds selectively to the extracellular domain of ErbB2 could eventually be useful as an anti-breast cancer therapy. To identify potential antagonists, the extracellular ligand binding domain of the ErbB2 is immobilized on a column support, and used to affinity purify cyclic peptides from oriented random peptide libraries. The structures of oriented peptide libraries are based on the primary sequences of the twelve known peptide ligands for other members of the ErbB receptor family. Amino acid residues conserved in the known ligands are preserved, while those that vary are made degenerate at their corresponding positions in the library to select for high affinity binding to ErbB2. We anticipate that a high affinity synthetic peptide ligand for ErbB2 will be identified by this approach, which will then be assessed for its ability to act as an antagonist for the receptor.

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Introduction

A fundamental mechanism by which cancer cells promote their own growth and malignancy is through the aberrant expression of polypeptide growth factors. These factors are believed to act by binding to specific cell surface receptor tyrosine kinases that are expressed by the same or neighboring tumor cells, triggering pathways that stimulate cellular growth through autocrine or paracrine mechanisms. ErbB2 is a 185 kDa cell surface transmembrane receptor tyrosine kinase that can mediate the growth or differentiation of a variety of cultured cells, and contributes to the development of cardiac and neural tissues of developing embryos. Its overexpression in a number of human tumors, including breast and ovarian tumors, correlates with an earlier patient relapse and a poor prognosis. The observation that the overexpression of ErbB2 stimulates its protein tyrosine kinase activity, together with the observation that activated alleles of the *erbB2/neu* gene induce metastatic tumors when introduced into murine mammary epithelium, suggest that the aberrant activation of ErbB2 kinase activity plays an active role in breast tumorigenesis and progression. Its potential role in breast cancer, as well as its accessible localization at the surface of tumor cells, has made ErbB2 an attractive target for the development of agents that might disrupt breast tumor progression.

A predominant theme in signal transduction, the propagation of extracellular signals to intracellular structures such as the cytoskeleton or nucleus, is that specific protein-protein interactions are formed and dissociated during the signaling process. These interactions are often mediated by a relatively large domain on one protein that recognizes a relatively short peptide motif on another. For example, src homology-2 (SH2) domains consist of ~100 amino acids, and recognize specific sequences of 4-6 amino acids immediately surrounding a phosphorylated tyrosine residue. Likewise, protein kinase domains generally consist of 350-400 amino acids but recognize and phosphorylate short amino acid motifs of 4-8 residues. During my post-doctoral work in Dr. Lewis Cantley's lab, I was involved in the development of a novel method for identifying short peptide motifs that bind to specific domains of intracellular signaling proteins. The specific work in which I was involved concerned the identification of preferred substrates of protein kinases using oriented peptide libraries. However, the technique has been applied successfully in the determination of motifs recognized by a variety of domains contained within intracellular signaling proteins, including SH2, SH3, LIM, PTB and PDZ domains.

The object of the method is to present a library of possible ligands to the domain whose specificity is unknown, and to allow the domain to select the ligands to which it binds with high affinity. This is achieved by expressing, purifying to homogeneity, and immobilizing the domain of interest to a support and passing a library of peptides degenerate at several positions over this column. After washing, bound peptides may be eluted from the column and sequenced to determine which residues at the degenerate positions are selected by the domain. The key to making these kind of experiments work, however, is having some prior knowledge as to the kinds of motifs that the domain of interest prefers. If a particular residue is known to be part of the recognized motif, such as phosphotyrosine for SH2 domains, that residue may be introduced as non-degenerate in the library, and the surrounding residues left degenerate. While the non-degenerate residue(s) serves as a primary determinate for binding, the surrounding degenerate residues may be selected on the basis of the specific preferences of the particular domain.

In the studies described here, we extend this approach to examine peptide motifs selected by the extracellular domains of receptors, for the specific and novel purpose of identifying potential antagonists for the ErbB2 receptor. An antagonist of ErbB2 could act to prevent the auto-activation of the receptor associated with overexpression by interfering with its ability to homodimerize. Antagonists could also interfere with ErbB2 activation by blocking the binding of its intrinsic ligand, if such a molecule exists, or by blocking the heterodimerization of ErbB2 with other ErbB receptors stimulated by heterologous EGF-like ligands. The EGF-like ligands for the ErbB receptors have a common structure: six characteristically-spaced cysteine residues together with several other highly conserved or invariant residues. This points to the possibility that these ligands may serve as a foundation upon which to build oriented peptide libraries for identifying ligands for ErbB2. Such ligands can then be screened for their potential antagonistic properties.

Report

Specific Aim I: Express the extracellular domain of ErbB2 (ErbB2-ECD) in milligram quantities and purify the expressed product to homogeneity. The overall strategy for this aim is to simultaneously express and characterize the ErbB2-ECD in insect cells and mammlian cells and characterize the expressed products. From the characterization, the system that yields the most active protein in a form that is most readily purified will be up-scaled for production of recombinant protein. It is estimated that milligram quantities of purified protein may be obtained within one year.

A) Transfection and expression of human ErbB2-ECD in insect cells and COS cells
   1. Subclone cDNA encoding the extracellular domain into expression vectors, sequence for PCR errors: months 1-3
   2. Transfection, selection of stably transfected COS cells and recombinant baculovirus, screen clones for ErbB2/Neu expression levels: months 3-5
   3. Characterization of the expressed products: months 6-7

B) Large scale production and purification of recombinant protein: months 8-12

We have expressed the ErbB2 extracellular domain in High Five insect cells as a glutathione-S-transferase (GST) fusion protein using baculovirus technology. The cDNA encoding human ErbB2 extracellular domain was amplified by polymerase chain reaction, subcloned into the insect cell transfer vector pAcSecG2T, and confirmed by sequencing. This material was used to generate recombinant baculovirus according to our standard protocols. Recombinant viruses were plaque purified, amplified and used to infect High Five insect cells to produce the recombinant protein. High Five cells are grown in the absence of serum, minimizing contamination from other proteins. This fusion protein was recognized by five out of five tested conformationally-sensitive antibodies to the ErbB2 extracellular domain , strongly suggesting that the expressed protein is functionally similar to that found in breast tumor cells. Our studies indicate that we can obtain 1 milligram of homogeneous expressed protein from five 150 mm dishes of High Five insect cells. Hence, the experiments of this specific aim have been successfully completed.

Specific Aim II: Develop an oriented peptide library approach to determine peptide motifs selected by the ErbB2-ECD. On the basis of the structures of the known EGF-like ligands, a series of oriented peptide libraries will be designed, synthesized, characterized and employed in peptide selection studies to obtain the optimal motif for binding to ErbB2-ECD. The success of each of the libraries using this method cannot be predicted in advance, and the design of subsequent libraries will depend on results obtained from selection studies with previous ones. Of the three aims this is by far the most challenging, and it is anticipated that this step will require up to one and a half years.

A) Synthesis, disulfide linking and characterization by mass spectrometry of first generation peptide libraries: may be carried out at the same time as receptor expression studies, months 1-12

B) Peptide selection studies and sequencing of first generation peptide libraries: months 13-14

C) Refinement of library designs from the results with first generation libraries, synthesis of new libraries, characterization, further peptide selection studies: months 15-30.

Our first synthesized library corresponded to the third disufide loop of the active EGF-like ligands, and had the primary structure The primary sequence of this initial library will thus read CXXXFXGXRC, where X represents a mixture of 19 amino acids excluding. We attempted to oxidize this material under a variety of conditions and found that at low peptide concentration, efficient cyclization was achieved with minimal formation of library aggregates. Non-cyclized material was removed using immobilized N-ethylmaleimide, which covalently reacts with free sulfhydryl groups. Figure 1 shows a mass spectrometry reading of our final purified cyclized material. On the basis of the area under each peak, we estimate that almost 70% of the material is monomeric cyclized peptide library, and we currently have 19 milligrams of the material.

Conclusions

We have prepared the initial two reagents necessary for the success of the project on schedule. We are able to produce milligram quantities of homogeneous active ErbB2-ECD by infection of High Five insect cells with recombinant baculovirus and purification of the recombinant protein by affinity for reduced glutathione. We are currently optimizing conditions for immobilizing this material to a solid support for screening the cyclic library. We have also produced the first oriented cyclic library, based on the structure of the third disulfide loop of EGF-like growth factors, cyclized it by oxidizing its two cysteines, purified non-cyclized peptides, and confirmed that the resulting material is predominantly monomeric cyclic library of the proper average molecular weight. In the coming year we will use the library to screen against the ErbB2-ECD, and hopefully obtain a motif to which this receptor binds with high affinity. If this is observed, this peptide will be synthesized and its potential antagonistic properties assessed. If not, a second generation of cyclic libraries will be produced and screened against ErbB2-ECD.

Tufts Protein Chemistry Facility

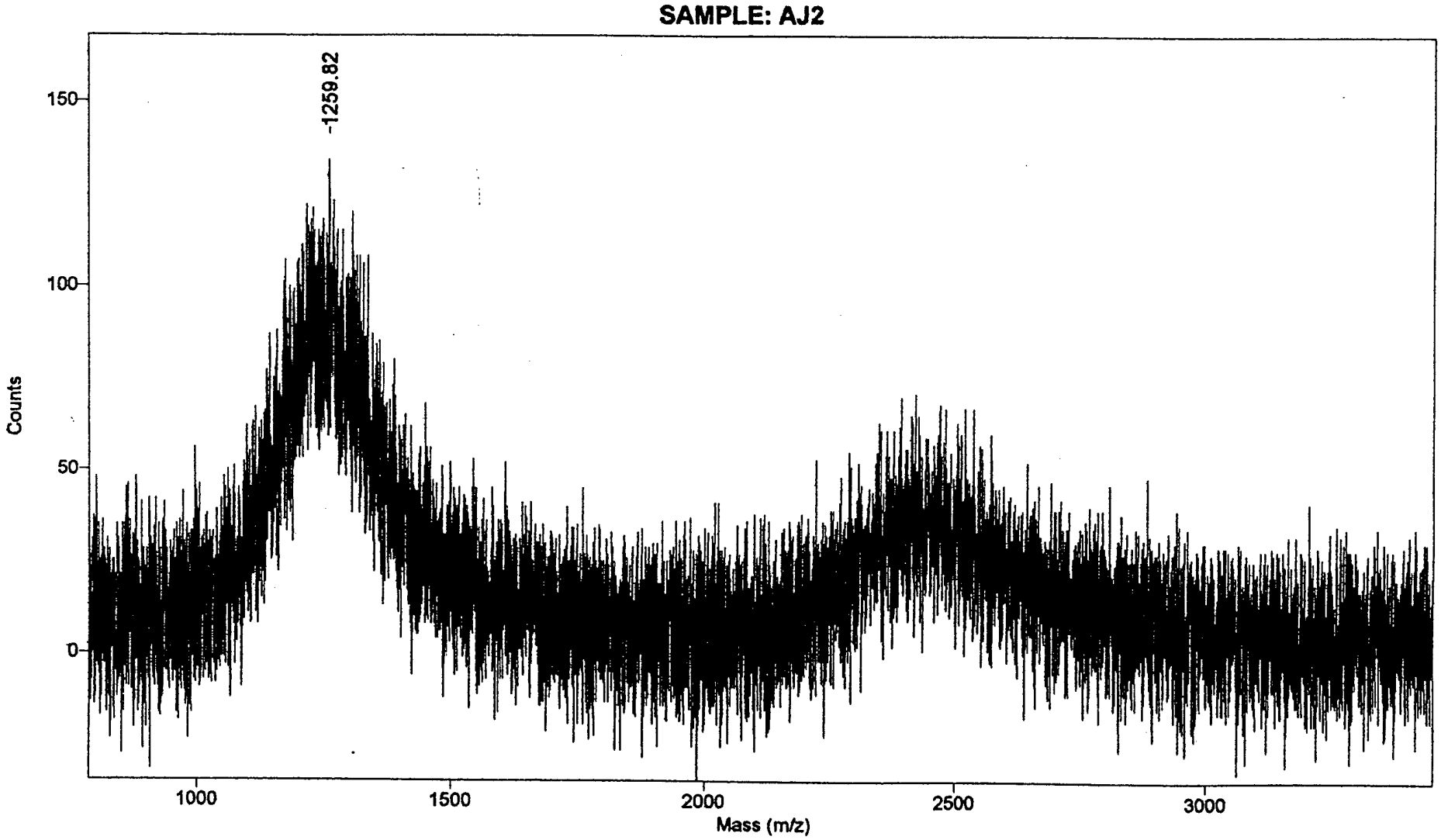

Figure 1

Comment: AJ2 CRUDE

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Collected: 5/26/98 8:46 AM | Negative Ions: OFF | Laser : 345 | Low Mass Gate: OFF

Acquired File:c:\voyager\data\AJ2.ms | File # 1 = C:\VOYAGER\DATA\AJ2.MS | Cal File:C:\VOYAGER\BRACTH.USR | Pressure: 4.15e-07